

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification<sup>4</sup> : <b>A61K 31/35</b></p>	<p><b>A1</b></p>	<p>(11) International Publication Number: <b>WO 89/ 07441</b> (43) International Publication Date: 24 August 1989 (24.08.89)</p>
<p>(21) International Application Number: PCT/US89/00516 (22) International Filing Date: 9 February 1989 (09.02.89) (31) Priority Application Number: 154,853 (32) Priority Date: 10 February 1988 (10.02.88) (33) Priority Country: US (71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 2199 Addison Street, Berkeley, CA 94720 (US). (72) Inventors: TSENG, Alexander, Jr. ; 1168 Greenwood Avenue, Palo Alto, CA 94301 (US). McLICK, Jerome ; 1292 Stanyan Street, San Francisco, CA 94117 (US). ALAEDDIN, Hakam ; 777 Morrell Avenue, #307, Burlingame, CA 94010 (US).</p>		<p>(74) Agents: SIEBERT, Suzanne, J. et al.; Majestic, Parsons, Siebert &amp; Hsue, Four Embarcadero Center, Suite 1450, San Francisco, CA 94111-4121 (US). (81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), DK, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).  <b>Published</b> <i>With international search report.</i> <i>With amended claims and statement.</i></p>
<p>(54) Title: 6-AMINO-1,2-BENZOPYRONE ANTITUMORIGENIC AGENTS AND METHOD</p> <p>(57) Abstract</p> <p>6-amino-1,2-benzopyrone is administered as a chemopreventative, or antitumorigenic agent, to inhibit tumor-igenicity in a mammalian host. Preferably, the administering is by a daily oral dose and provides about 100 mg per day.</p> <p><b>BEST AVAILABLE COPY</b></p>		

***FOR THE PURPOSES OF INFORMATION ONLY***

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	ML	Mali
AU	Australia	GA	Gabon	MR	Mauritania
BB	Barbados	GB	United Kingdom	MW	Malawi
BE	Belgium	HU	Hungary	NL	Netherlands
BG	Bulgaria	IT	Italy	NO	Norway
BJ	Benin	JP	Japan	RO	Romania
BR	Brazil	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	LI	Liechtenstein	SN	Senegal
CH	Switzerland	LK	Sri Lanka	SU	Soviet Union
CM	Cameroon	LU	Luxembourg	TD	Chad
DE	Germany, Federal Republic of	MC	Monaco	TG	Togo
DK	Denmark	MG	Madagascar	US	United States of America
FI	Finland				

6-AMINO-1,2-BENZOPYRONE ANTITUMORIGENIC  
AGENTS AND METHOD

Inventors: ALEXANDER TSENG, JR., JEROME McLICK and  
ALAEDDIN HAKAM

5    Field of the Invention

The present invention generally relates to anti-tumorigenic agents, and more particularly to use of 6-amino-1,2-benzopyrone, or 6-aminocoumarin, and salts thereof in inhibiting tumor growth in a mammalian host.

10   Background of the Invention

Some populations are at high risk of developing tumors. For example, immunosuppressed patients, such as post-cardiac or renal transplant patients, can experience a high tumor incidence. Similarly, AIDS (Acquired Immune  
15   Deficiency Syndrome) patients or persons with the ARC (AIDS Related Complex) syndrome are another patient group in which tumors are prone to develop.

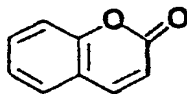
One approach in attempts to inhibit tumor growth is to administer a phenylalkylamine or the like as a calcium  
20   channel blocker compound, as described by U.S. Patent 4,690,935, inventors Taylor et al., issued September 1, 1987. Another approach is described by U.S. Patent 4,714,683, inventor Shoyab, issued December 22, 1987 in which administration of a certain polypeptide is said to

retard the growth of neoplastic cells. U.S. Patent 4,684,627, inventor LeVeen, issued August 4, 1987, describes administering compounds, such as lonidamine and phlorizin, to humans with lung cancer, which compounds  
5 are said to inhibit glucose transport in cancer cells.

Poly(ADP-ribose) polymerase has been implicated as an enzyme system involved in neoplastic transformation. 1,2-benzopyrone (frequently referred to as coumarin) has recently been reported to inhibit poly(ADP-ribose) polymerase at the DNA binding site of the enzyme. Although  
10 poly(ADP-ribose) polymerase was inhibited by 1,2-benzopyrone to the same extent in both non-transformed and neoplastic (transformed) cells, the arrest of cell proliferation occurred only in transformed cells expressing oncogenes. Tseng, Jr. et al., "Prevention of Tumorigenesis of Oncogene-Transformed Rat Fibroblasts with DNA Site Inhibitors of Poly(ADP Ribose) Polymerase,"  
15 Proc. Natl. Acad. Sci. USA, Volume 84, pp. 1107-1111 (February 1987).

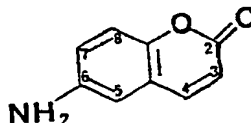
20 1,2-benzopyrone has the structure illustrated by Formula I, below.

FORMULA I



Summary of the Invention

In one aspect of the present invention, a method for inhibiting tumorigenicity in a patient comprises administering to the patient an effective amount of a compound  
5 having the formula



or a therapeutically acceptable salt, such as chloride or sulfate, thereof. Said compound, designated 6-amino-1,2-benzopyrone, or 6-aminocoumarin, is surprisingly more efficacious than the unsubstituted, 1,2-benzopyrone  
10 compound in inhibiting growth of the human renal cancer cell line CRL1611. The compound (like 1,2-benzopyrone) is readily administered, such as by oral doses, and is non-toxic. The compound is believed particularly useful in mammalian hosts, or patients, who are at risk due to  
15 exposure to mutagenic agents or are particularly susceptible to neoplastic formation. Thus, use of the 6-amino-1,2-benzopyrone compound and its pharmaceutically acceptable salts is particularly preferred as a chemopreventative agent for populations at high risk of develop-  
20 ing tumors. By chemoprevention is meant the use of chemical agents to inhibit, block or reverse the growth and/or progression of cancer.

Brief Description of the Drawings:

Figure 1 is a schematic representation of the 4.8 Kb  
25 EJ-ras oncogene fragment and pCV108 cloning vector; and,

Figure 2 is a graph showing the effect of 6-amino-1,2-benzopyrone on the initial velocity of auto-mono-ADP-ribosylation of the enzyme as a function of varying concentrations of octameric DNA.

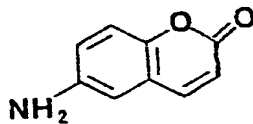
5 Detailed Description of the Preferred Embodiments

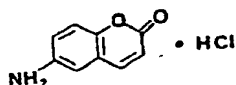
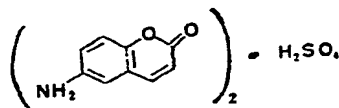
The compound 1,2-benzopyrone has been found clinically effective in several metastatic human tumors, i.e., metastatic melanoma and metastatic renal cell cancer, when used in combination with cimetidine.

10 Practice of the present invention includes use of 6-amino-1,2-benzopyrone or its salts as a chemopreventative, or antitumorigenic, agent. The compound may be synthesized in good yield and converted to a therapeutically acceptable salt, preferably such as sulfate  
15 and chloride, most preferably as the chloride salt. These salts improve the solubility of the free base and are shelf-stable (e.g., are non-hygroscopic and thermally stable at ambient temperatures). The structure of the free base 6-amino-1,2-benzopyrone is illustrated by  
20 Formula IIA and that of two preferred salts by Formulas IIB and IIC. The compound is obtained as the free base and can be readily converted to salts with inorganic acids, but does not form salts with weaker organic acids such as acetic acid.

25

FORMULA IIA



FORMULA IIBFORMULA IIC

A number of analogs to the Formula IIA compound are known in the literature which may have similar pharmacological properties. These analogs include 8-amino-1,2-benzopyrone, 3-amino-1,2-benzopyrone, 6-amino-3-hydroxy-1,2-benzopyrone, and 8-amino-3-hydroxy-1,2-benzopyrone.

A preferred method for synthesizing the Formula IIA, IIB and IIC antitumorigenic agents will now be described.

10 Preferred Synthesis Method

15 6-amino-1,2-benzopyrone: To a stirred suspension of 0.50 gm of 10% palladium on activated carbon in 30 ml of degassed water was added a solution of potassium borohydride (2.70 gm, 0.050 mol) in water (35 ml). A solution of 6-nitro-1,2-benzopyrone (3.82 gm, 0.020 mol; available from Pflatz & Bauer) in methanol (1000 ml) was then slowly added (10 minutes) to the stirred mixture at ambient temperature. After stirring for an additional 15 minutes, the mixture was filtered through a bed of Celite to remove the catalyst, and the filtrate was then stripped of methanol by rotary evaporation. The damp residue was suspended in cold water (100 ml) and collected by suction filtration. The crude yellow product was washed on the filter with additional cold water and then dried and

20

weighed (2.83 gm). The material was finally recrystallized from ethanol to afford 2.32 gm (72% yield), m.p. 166-169°C (lit 166-167°C, see Kokotos et al., J. Heterocyclic Chem., 23, p. 87 (1986). MASS: 161 ( $M^+$ ), 133, 104, 78, 52.

6-amino-1,2-benzopyrone hydrochloride: To a chilled slurry of 6-amino-1,2-benzopyrone (1.50 gm, 0.009 mol) in 20 ml of water was added 2 M HCl dropwise in sufficient quantity with stirring to dissolve the base, yielding a yellow solution. The solution was filtered to remove a small amount of cloudiness and then stripped of water by rotary evaporation. The residue was crystallized from 1/1 (v/v) methanol/ethanol to give 1.59 gm (86% yield) of pale yellow prisms, m.p. 280-285°C with decomposition (the material darkens above 230°C).

Elemental analysis for  $C_9H_8ClNO_2$ : Calculated: C, 54.70; H, 4.08; Cl, 17.94; N, 7.09. Found: C, 54.80; H, 4.09; Cl, 18.06; N, 6.98. MASS: 161 ( $M-HCl$ )<sup>+</sup>, 133, 104, 78, 52, 51.

6-amino-1,2-benzopyrone sulfate: To a chilled, stirred slurry of 6-amino-1,2-benzopyrone (0.48 gm, 0.003 mol) in 6 ml of water was added 2M  $H_2SO_4$  dropwise in sufficient quantity to dissolve the base. The solution was filtered to remove cloudiness and then stripped of water by rotary evaporation. The residue, which contained a small amount of excess  $H_2SO_4$ , was dissolved in hot ethanol, which upon cooling gave tan-colored platelet crystals. The material was collected, washed with cold solvent, and crystallized again from ethanol to afford 0.38 gm (60% yield) of the salt, m.p. 214-215°C with decomposition.



Elemental analysis for  $(C_9H_8NO)_2SO_4$ : Calculated: C, 51.43; H, 3.84; N, 6.66; S, 7.63. Found: C, 51.40; H, 3.38; N, 6.43; S, 7.66.

---

5       The antitumorigenic properties of the 6-amino-1,2-benzopyrone compound, including its growth inhibitory effects (expressed as  $IC_{50}$ ) and non-toxic concentrations (expressed as  $IC_0$ ) are illustrated by a variety of in vitro and in vivo assays. Thus, Example I describes  
10 measurement of the growth inhibitory effects in vitro with a rat fibroblast cell line designated 14C, and Example VI describes the in vivo tumor growth inhibitory effects of the Formula II compound in salt form.

15       The 14C cell line appears morphologically similar to the parent cell line (Rat-1 fibroblasts) in the absence of dexamethasone in the culture medium. The cells appear flat and are non-refractive to light. After a 24 hour exposure to 0.1  $\mu$ M dexamethasone, the cells round up and develop cellular projections, heap up into refractile  
20 foci, and lose their contact growth inhibition.

25       The 14C cell line spontaneously forms few and only very small colonies in soft agar, but exposure to 0.1  $\mu$ M dexamethasone (Dex) greatly increases both the number and size of colonies. Dexamethasone is a potent synthetic corticosteroid. The 14C cell line contains a steroid-inducible EJ-ras oncogene isolated from a human bladder carcinoma. The pMTV-EJras plasmid was made by placing the EJ-ras coding domain under the transcriptional control of the mouse mammary tumor virus (MTV; steroid-inducible) promoter/enhancer, and it also contains a  
30

neomycin-resistance gene (see Figure 1). EJ-ras is a mutant c-Ha-ras gene isolated from the EJ/T24 bladder carcinoma cell lines. The steroid-responsive portion of the MTV long terminal repeat (Cla I-BamHI fragment) from plasmid p484 was subcloned between the above restriction sites in the vector pCV108, which carries the neomycin-resistance gene, generating pMTV108. A fragment of the pEJ6.6 plasmid [the 4.8-kilobase (kb) fragment from a Sma I site located approximately 20 base pairs (bp) upstream from the initiation ATG to the Bgl II site near the 3' end of the clone] was converted to Bgl II ends with linkers and inserted into the BamHI site of pMTV108, generating pMTV-EJras. Rat-1 fibroblasts were transfected with this plasmid by the calcium phosphate technique and selected in the presence of 400 µg/ml of G418 (GIBCO). Drug-resistant colonies were isolated with the aid of a cloning cylinder.

Because the 14C cell line contains the inducible EJ-ras oncogene, the effect of compounds described in this invention can be determined before and after overt transformation and avoid biochemical complications introduced by the toxicity of chemical carcinogens and irradiation.

#### EXAMPLE I

##### Growth Assay in 14C Cells

Cell cultures (of the 14C cell line previously discussed) were initiated at a cell density of 20,000 cells per T25 culture flasks, and grown in Dulbecco's modified Eagles's medium (DMEM) supplemented with 10% fetal calf serum, 0.5% penicillin/streptomycin, and 2mM

glutamine. The flasks were incubated at 37°C for 24 hours to allow the cells to attach to the plastic.

The 6-amino-1,2-benzopyrone in free base and in chloride salt form was then added and cell counts were performed after 24, 48 and 72 hours of further incubation. The cells were trypsinized (0.25% STV trypsin) and cell counts were determined on a Coulter Counter (ZBI model).

Table I shows the growth inhibitory effects of 6-amino-1,2-benzopyrone and its salts on 14C cells exposed to the drug for 72 hours.

TABLE I  
Growth Inhibition Assay of 14C Cells

	<u>IC<sub>50</sub></u>	<u>Maximal IC<sub>(0)</sub></u>
6-aminocoumarin	1mM	100μM
15 6-aminocoumarin·HCl	2mM	100μM

This growth inhibition assay shows that an inhibitory concentration of drug can be defined, as well as a maximal, non-inhibiting concentration. When the maximal, non-inhibitory concentration of 6-aminocoumarin (that is, 6-amino-1,2-benzopyrone) is used in the soft agarose and tumorigenic assays, the drug proves to be effective in preventing growth. Thus, a non-toxic drug concentration for preventing the tumorigenic phenotype can be defined and is set out in Table III.

EXAMPLE II  
Growth Assay in Human Cells

5 The growth inhibitory effects of the antitumorigenic agent in accordance with the invention was also measured in human renal carcinoma cell lines (CRL1611 and KEC-1) and a human bladder carcinoma cell line (253J). The 253J and KEC-1 cell lines were as described by Williams, Invest. Urol., 17, pp. 359-363 (1980). The CRL1611 cell line was obtained from the ATCC. The results were similar to those described in Example I, and are set forth in Table II.

TABLE II  
Growth Inhibition at 72 Hours

<u>253J (Human Bladder Cancer Cell Line)</u>		
15	6-aminocoumarin·HCl	$IC_{50} = 4.5 \times 10^{-4} M$
	6-aminocoumarin	$IC_{50} = 10^{-6} M$
<u>KEC-1 (Human Renal Cancer Cell Line)</u>		
	6-aminocoumarin	$IC_{80} = 10^{-4} M$
<u>CRL1611 (Human Renal Cancer Cell Line)</u>		
20	6-aminocoumarin	$IC_{85} = 10^{-4} M$

EXAMPLE IIISoft Agarose Assay with <sup>14</sup>C Cells

Assays were conducted to measure anchorage-independent colony formation of cells when plated into soft agarose. 25,000 <sup>14</sup>C cells were initiated into T25 culture flasks and exposed for 48 hours to either 0.1  $\mu$ M dexamethasone (Dex) or to sterile water. The <sup>14</sup>C cells were then exposed to a four log concentration of 6-amino-1,2-benzopyrone for an additional 72 hours. Cells ( $1 \times 10^3$ ) were suspended in 8 ml of 0.35% agarose (Calbiochem, type A) in DMEM containing 10% fetal calf serum and overlaid above 5 ml of 0.7% agarose in DMEM containing 10% fetal calf serum in 60 mm petri dishes. Following incubation at 37°C for 10 days, the cells were stained with 1 ml of MTT dye (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma) overnight and counted. The number of colonies measuring over 0.3 mm in diameter (greater than 100 cells) was counted under low power phase microscopy.

The effects of 6-amino-1,2-benzopyrone on colony formation (and of 1,2-benzopyrone as comparison) are illustrated by the data of Table III.

TABLE III

	Treatment	No. of Colonies	IC <sub>50</sub>
	<u>Rat-1 Fibroblasts (Control)</u>		
	(-) Dex	10 $\pm$ 4	--
5	<u>14C Cells (Control)</u>		
	(-) Dex	42 $\pm$ 48	--
	(+) Dex	308 $\pm$ 69	--
	<u>1,2-benzopyrone at 0.1 mM</u>		
	(-) Dex	56 $\pm$ 53	0.5 mM
10	(+) Dex	74 $\pm$ 18	0.5 mM
	<u>6-amino-1,2-benzopyrone at 0.1 mM</u>		
	(-) Dex	34 $\pm$ 12	1 mM
	(+) Dex	33 $\pm$ 7	1 mM
	<u>6-amino-1,2-benzopyrone·HCl at 0.1 mM</u>		
15	(-) Dex	43 $\pm$ 33	2 mM
	(+) Dex	49 $\pm$ 33	2 mM
	<u>6-amino-1,2-benzopyrone·HCl at 1 mM</u>		
	(-) Dex	46 $\pm$ 12	2 mM
	(+) Dex	29 $\pm$ 6	2 mM

20 As may be seen from the data of Table III, the control cells were transformed by being induced with dexamethasone, but treatments with 6-amino-1,2-benzopyrone or treatments with 6-amino-1,2-benzopyrone·HCl inhibited such dexamethasone induced growth at concentra-

tions as small as 0.1 mM. As may also be seen, the related compound 1,2-benzopyrone also had an inhibitory effect, but one tending to be less efficacious.

#### EXAMPLE IV

##### 5     Soft Agarose Assays with Human Cancer Cells

Soft agarose assays were performed as described in Example III, but with CRL1611 human renal carcinoma cells; however, there was no exposure to dexamethasone (since the cells are already transformed) and  $10^5$  cells were used. The assays were conducted with varying concentrations of 1,2-benzopyrone and 6-amino-1,2-benzopyrone, and the results are set out in Table IV.

TABLE IV

		<u>Number of Colonies</u>
15	Control	382 ± 85
	$10^{-4}$ M 1,2-benzopyrone	244 ± 85
	$10^{-5}$ M 1,2-benzopyrone	315 ± 43
	$10^{-6}$ M 1,2-benzopyrone	402 ± 21
	$10^{-4}$ M 6-amino-1,2-benzopyrone	175 ± 35
20	$10^{-5}$ M 6-amino-1,2-benzopyrone	170 ± 8
	$10^{-6}$ M 6-amino-1,2-benzopyrone	170 ± 37

As may be seen from the data of Table IV, the 6-amino-1,2-benzopyrone treatment at only 1  $\mu$ M ( $10^{-6}$  M) was strikingly more effective in inhibiting colonies of the human renal cell cancer line than was the 1,2-benzopyrone compound at 100 times the concentration.

EXAMPLE V  
Cell Cycle Assay

Early passage nonsynchronized 14C cells were pre-treated with 0.1  $\mu$ M dexamethasone for 72 hours to induce transformation. This was followed by either 24 hour treatment with 100  $\mu$ M 6-amino-1,2-benzopyrone or phosphate buffer saline. Non-dexamethasone transformed cells were similarly treated with 100  $\mu$ M 6-amino-1,2-benzopyrone or with phosphate buffer saline only. Media and drugs were changed daily. When cells reached 70% confluency in T75 flasks, they were pulse labeled with 15  $\mu$ M 5-bromodeoxyridine (BrdUrd) for 30 minutes, washed with PBS, trypsinized, centrifuged at 4°C and fixed in 70% cold ethanol. The fixed cells were pelleted, resuspended and incubated for 15 minutes in 2.5 M HCl/0.7% TX-100. The cells were then washed in PBS and 0.5% Tween 20. Mouse anti-BrdUrd monoclonal antibody IU-4 diluted 1:4000 in PBS/0.5% Tween 20/0.5% Carnation Milk powder was then incubated with cells for 30 minutes at room temperature. Cells were washed and treated with FITC-conjugated rabbit anti-mouse IgG diluted 1:150 in PBS/Tween 20/milk powder and incubated for 20 minutes at room temperature. Cells were washed and stained with 10  $\mu$ g/ml of propidium iodide for 30 minutes. Cells were filtered through a 27  $\mu$ M Nitrex mesh and 20,000 cells were analyzed on a FACS II flow cytometer (Becton-Dickenson, Mountain View, CA). Cells were excited at 488 nm. Red fluorescence from propidium iodide was collected through a 600 nm long pass filter and recorded on linear scale as a measure of DNA content. Green fluorescence from fluorescein was collected through a 514 nm band pass filter and recorded after logarithmic amplification as a measure of the



amount of incorporated BrdUrd. List mode data was collected with Electric Desk software and displayed as dual parameter correlated data. The percentage of cells in  $G_0/G_1$ ,  $G_2/M$  and S phases of the cell cycle was calculated within a 5% error by appropriate gating around those easily defined populations.

The cell cycle effects of the 6-amino-1,2-benzopyrnone treatment and the control are illustrated by the data of Table V.

10

TABLE V

	<u><math>G_0/G_1</math></u>	<u>S</u>
Control	67%	21%
0.1 $\mu$ M Dex	55%	31%
100 $\mu$ M 6-amino- 1,2-benzopyrnone	65%	18%
0.1 $\mu$ M Dex and 100 $\mu$ M 6-amino-1,2-benzopyrnone	61%	19%

15

20

As may be seen from the data in Table V, dexamethasone increases the percentage of cells in the S phase with a concomitant decrease in the percentage of cells in the  $G_0/G_1$  phase of the cell cycle. This represents a recruitment of cells from the  $G_0/G_1$  into the S phase. Treatment with 6-amino-1,2-benzopyrnone of  $^{14}C$  cells that

have not been transformed by dexamethasone has no significant effect on the percentage of cells in these phases compared to control. However, treatment of dexamethasone transformed 14C cells with 6-amino-1,2-benzopyrone reverses the effect of dexamethasone.

As may also be seen from the data of Table V, the 6-amino-1,2-benzopyrone treatment seems to have specificity in causing a cell cycle block in the  $G_0/G_1$  phase of transformed cells.

10

#### EXAMPLE VI

##### Tumorigenesis Inhibition in Vivo

An in vivo tumorigenesis assay using 14C cells was performed. Subcutaneous injections of  $10^5$  cells in Fischer 344 rats (male, 150-g body weight) led to the development of lethal fibrosarcomas. Groups of at least ten animals were tested per group. Tumor incidence and weight were compared between four different treatment groups, as shown in Table VI. Two groups of animals were injected with 14C cells that were pretreated with 100  $\mu$ M 6-amino-1,2-benzopyrone·HCl in vitro, versus two groups that were injected with 14C cells that had not been pretreated. Two groups of animals received 6-amino-1,2-benzopyrone HCl orally as administered in their drinking water, versus two groups that received sterile water only. In the drug-treated groups, each bottle contained 200 cc of 20  $\mu$ M 6-amino-1,2-benzopyrone·HCl dissolved in sterile water. The drinking bottles were refilled every three days and monitored for the volume of water consumed. The animals were weighed and survivors were recorded.

Animals were sacrificed two weeks after inoculation of 14C cells and tumor weights are measured. The data of Table VI summarizes the results.

TABLE VI

Group	100 $\mu$ M 6-1,2-amino-benzopyrone Pre-treatment in Vitro	200 $\mu$ M 6-amino-1,2-benzopyrone in Water Supply	No. of Injections (No. of Rats)	No. Tumors/Inj. (>1gm)	Average Tumor Weight
1.	(-)	(-)	75 (38)	50/75 (66%)	3.5 $\pm$ 2.5 gm
2.	(+)	(-)	20 (10)	2/20 (10%)	0.34 $\pm$ 0.78 gm
3.	(-)	(+)	20 (10)	3/20 (15%)	0.66 $\pm$ 1.68 gm
4.	(+)	(+)	22 (11)	1/22 (5%)	5.11 $\pm$ 0.49 gm

As may be seen from the data of Table VI, the pretreatment of the transformable 14C cells with 6-amino-1,2-benzopyrone was effective in inhibiting both the number of tumors and tumor weight. Similarly, the dietary administration of 6-amino-1,2-benzopyrone was also effective in reducing the number of tumors and the average tumor weight. Best results were obtained with both pretreatment and oral administration of 6-amino-1,2-benzopyrone in reducing the number of tumors and reducing tumor weight.

It was calculated that a 200  $\mu$ M drug concentration in the drinking water for seven days could maintain a cellular concentration of unmetabolized drug between 50-88  $\mu$ M, that is, in the same order of magnitude as the competitive  $K_i$  obtained with 6-amino-1,2-benzopyrone with purified poly(ADP-ribose) polymerase enzyme.

EXAMPLE VIIInhibition of Poly(ADP-ribose) Polymerase

The 6-amino-1,2-benzopyrone compound competitively inhibits the DNA binding site of poly(ADP-ribose) polymerase (also known as adenosine diphosphoribosyl-transferase, EC 2.4.30), which is a nuclear enzyme that catalyzes the cleavage of oxidized NAD with the concomitant covalent attachment of ADP-ribose to acceptor nuclear proteins. As described below, the  $K_i$  (inhibitory constant) is 28  $\mu\text{M}$  for 6-amino-1,2-benzopyrone. By comparison, the  $K_i$  for 1,2-benzopyrone is 47  $\mu\text{M}$ .

The 6-amino-1,2-benzopyrone compound is a potent inhibitor of the enzyme adenosine diphosphoribosyl-transferase. Specifically, the inhibitory effect of 6-amino-1,2-benzopyrone on the initial velocity of mono-ADP-ribosylation of the enzyme as a function of the concentration of octameric coenzymic DNA was studied. Figure 2 shows a double-reciprocal plot of the data obtained. The graphical results demonstrate that 6-amino-1,2-benzopyrone competitively inhibits at the coenzymic DNA binding site with an apparent  $K_i$  of 28  $\mu\text{M}$ . (Without the inhibitor, at a fixed NAD concentration a Michaelis-Menten relationship exists between the initial velocity and the concentration of coenzymic DNA (lowest curve in the figure) with an apparent binding constant of 1  $\mu\text{M}$ . This value was obtained by varying the concentration of the octameric DNA over a 10-fold range.) The results establish that a site exists which is structurally unrelated to NAD at which the inhibitor binds.

The octameric DNA used in this study was 5'-A-G-A-T-C-A-G-T-3' in the duplex form with its complement. The initial velocity rates were determined using the following enzyme incubation buffer: 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 20 mM MgCl<sub>2</sub>, 2.5 mM dithiothreitol, and 7 mM NaCl. NAD concentration was 25 nM and the final volume of each assay was 100 uL. The reaction was started by addition of 1 µg of enzyme, and incubation was carried out at 24°C for 2 minutes and then stopped by addition of 20% aqueous trichloroacetic acid. The amount of product ADP-ribose formed was determined by collection on Whatman glass microfiber filters.

---

The implications of the tumorigenesis inhibition illustration of Example VI are that administration of 6-amino-1,2-benzopyrone or its salts will be useful with adjuvant therapy in treating different types of cancers. For example, among the present treatments following breast cancer surgery are adjuvant uses of methotrexate, 5-fluorouracil, doxorubicin, cyclophosphamide, vincristine, and prednisone. Patients with advanced Kaposi's sarcoma are treated with vinblastine or tenoposide, but early staged Kaposi's sarcoma is being treated with adjuvant azothymidine (AZT). It is contemplated that 6-amino-1,2-benzopyrone or its salts may be formulated with such other standard cytotoxic cancer therapies, as well as with various excipients or carriers, where such other ingredients are pharmaceutically acceptable. For example, an illustrative tablet formation for the oral administration of 6-amino-1,2-benzopyrone or its salts may include microcrystalline cellulose, dicalcium phosphate (dihydrate), stearic acid, magnesium stearate,

silica, flavorings and the like tableting components known to the art.

5 The dosage level administered in vivo to a human patient will generally range from about 1 to about 100 mg orally per day, preferably about 100 mg orally per day. This dosage is effective in inhibiting tumors, but is believed to be substantially nontoxic to the patient. A two-year baboon study of the 1,2-benzopyrone compound revealed no liver damage when fed at 67.5 mg per kg baboon  
10 body weight per day.

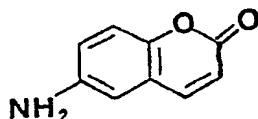
The dosage of compositions useful for the present invention may take a variety of forms, such as a solid tablet, or may be formulated with appropriate physiologically acceptable additives as a powder, dispersion or  
15 solution. Particularly if used as a solution, the antitumorigenic agent is preferably in salt form to increase solubility, and an agent such as cimetidine (which inhibits cytochrome P-450) may be included to reduce the rate by which the 6-amino-1,2-benzopyrone or  
20 salt thereof is metabolized by the liver in the body.

Although the present invention has been described with reference to specific examples, it should be understood that various modifications and variations can be easily made by those skilled in the art without departing  
25 from the spirit of the invention. Accordingly, the foregoing disclosure should be interpreted as illustrative only and not to be interpreted in a limiting sense. The present invention is limited only by the scope of the following claims.

IT IS CLAIMED:

1. A method for inhibiting tumorigenicity in a mammalian host comprising:

administering to the host an effective amount  
5 of a compound having the formula



or a therapeutically acceptable salt thereof.

2. The method as in claim 1 wherein the administering is by an oral dose.

3. The method as in claim 2 wherein the oral dose includes the compound or salt thereof in sufficient amount to provide about 100 mg of the compound per day.

4. The method as in claim 1 wherein the effective amount is substantially non-toxic to the host.

5. An antitumorigenic agent having the formula



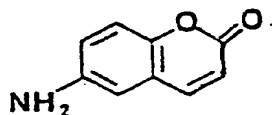
6. The antitumorigenic agent as in claim 5 combined with a pharmaceutically acceptable carrier or excipient.

## AMENDED CLAIMS

[received by the International Bureau  
on 24 July 1989 (24.07.89);  
original claims 5 and 6 cancelled; claims  
1 and 3 amended; new claims 7-10 added;  
other claims unchanged (4 pages)]

1. A method for inhibiting tumorigenicity in a mammalian host at risk of developing tumors comprising:

administering to the host an amount of a compound having the formula



or a therapeutically acceptable salt thereof, the amount administered being effective to inhibit tumor growth by itself or in conjunction with adjuvant therapy of renal carcinomas, bladder carcinomas, fibrosarcomas, breast carcinomas, prostate tumors, melanoma or Kaposi's sarcoma.

2. The method as in claim 1 wherein the administering is by an oral dose.

3. The method as in claim 2 wherein the oral dose includes the compound or salt thereof in sufficient amount to provide from about 1 mg to about 100 mg of the compound per day.



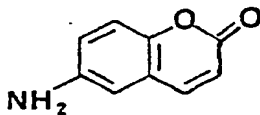
4. The method as in claim 1 wherein the effective amount is substantially non-toxic to the host.

5. Claim 5 is cancelled.

6. Claim 6 is cancelled.

7. A method for inhibiting tumorigenicity in a mammalian host at risk of developing renal carcinomas comprising:

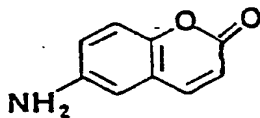
administering to the host an amount of a compound having the formula



or a therapeutically acceptable salt thereof, the amount administered being effective to inhibit growth of renal carcinomas by itself or in conjunction with adjuvant therapy therefore.

8. A method for inhibiting tumorigenicity in a mammalian host at risk of developing breast carcinomas comprising:

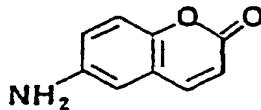
administering to the host an amount of a compound having the formula . . .



or a therapeutically acceptable salt thereof, the amount administered being effective to inhibit growth of breast carcinomas by itself or in conjunction with adjuvant therapy therefore.

9. A method for inhibiting tumorigenicity in a mammalian host at risk of developing prostate tumors comprising:

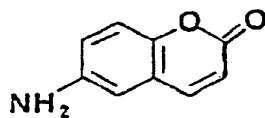
administering to the host an amount of a compound having the formula



or a therapeutically acceptable salt thereof, the amount administered being effective to inhibit growth of prostate tumors by itself or in conjunction with adjuvant therapy therefore.

10. A method for inhibiting tumorigenicity in a mammalian host at risk of developing melanomas comprising:

administering to the host an amount of a compound having the formula



or a therapeutically acceptable salt thereof, the amount administered being effective to inhibit growth of melanomas by itself or in conjunction with adjuvant therapy therefore.

## STATEMENT UNDER ARTICLE 19

This Statement is filed pursuant to Article 19(1) of the PCT and accompanies the amendment of the claims. As shown in the replacement sheets, claims 1 and 3 are changed, claims 2 and 4 remain unchanged, claims 5 and 6 are cancelled, and new claims 7, 8, 9 and 10 are added. The application now only asserts method claims for inhibiting tumorigenicity in mammalian hosts at risk of developing tumors/cancers. The inventive compound in each claim is administered by itself or in conjunction with adjuvant therapy for specific tumors or cancers. Claims for the inventive compound *per se* (i.e., claims 5 and 6) have been cancelled.

The amendment is in response to the international search report's Chemical Abstracts reference that cited an article by Kitagawa et al., published in Yakugaku Zasshi 83, 1124-8 (1963). The article disclosed pharmacological studies of coumarin derivatives, including 6-aminocoumarin-HCl, a salt of the inventive compound. The article indicated that 6-aminocoumarin-HCl exhibited general pharmacological activities. However, apparently no data regarding its effects on cancer/tumor were reported.

1 / 1

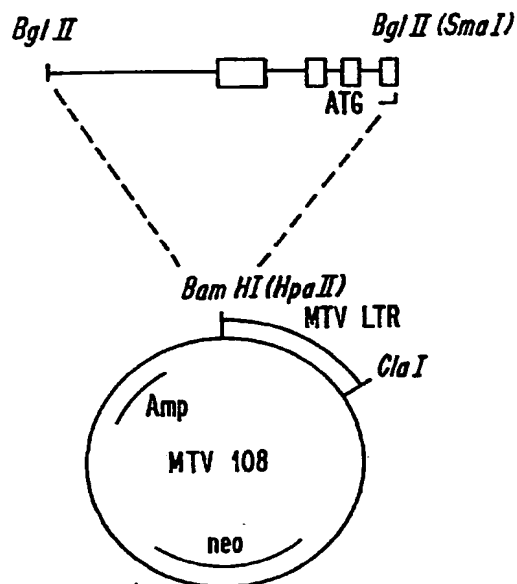
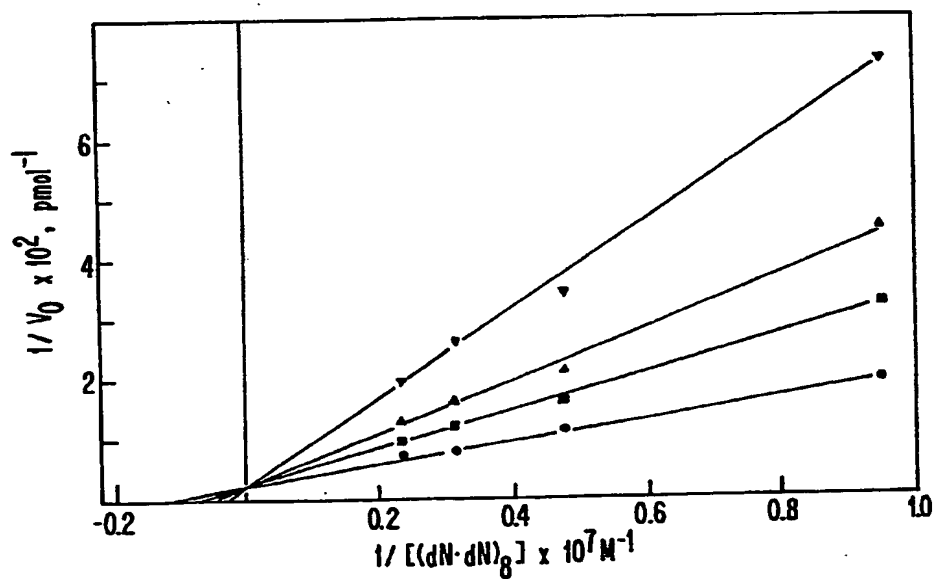


FIG. 1.

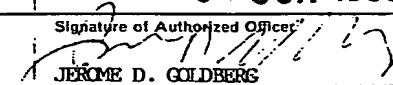


NO INHIBITOR PRESENT (•), 20  $\mu\text{M}$  (■), 40  $\mu\text{M}$  (▲), AND 80  $\mu\text{M}$  (▼) 6 AMINO-1,2-BENZOPYRONE.

FIG. 2.

# INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US89/00516**

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC <b>IPC(4): A61K 31/55</b> <b>U.S.Cl.: 514/457</b>		
<b>II. FIELDS SEARCHED</b> Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
U.S.	514/457	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup> <b>CHEMICAL ABSTRACTS - CHEMICAL SUBSTANCE 1907-1988 "2H-Bensopyran-2-cn<sub>3</sub>, 6-amino;"</b> <b>"coumarin, 6-amino".</b>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup></b>		
Category <sup>9</sup>	Citation of Document, <sup>11</sup> with Indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	Chemical Abstracts, Volume 60, Abstract No. 15017c (KITAGAWA ET AL)., "Coumarin derivatives for medical purposes" (1964).	1-6
<p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search <b>22 MAY 1989 (22.05.89)</b>		Date of Mailing of this International Search Report <b>07 JUN 1989</b>
International Searching Authority <b>ISA/US</b>		Signature of Authorized Officer  <b>JEROME D. GOLDBERG</b>

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**